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TNO report

TNO-DV2 2005 A222

Fast detection of ciprofloxacin resistance - Part II

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Snelle detectie van ciprofloxacineresistentie - deel II

Het onderzoek beschrijft een nieuwe analysemethode om eventueel aanwezige resistentie tegen een veel gebruikt antibioticum snel (binnen 1 uur) te herkennen. Wanneer dit het geval is bij een geconstateerde bacteriële infectie, kan vroegtijdig een ander antibioticum worden voorgeschreven, waarmee het ziekteproces aanmerkelijk verkort zal worden en mogelijk levens gered kunnen worden.



Beschrijving van de werkzaamheden

De eerder ontwikkelde methode werd aangepast en gevalideerd voor een aantal andere pathogenen, waaronder *Brucella melitensis* (veroorzaker van Brucellose), *Yersinia pestis* (veroorzaker van Pest) en *Staphylococcus aureus* (inclusief MRSA-stammen).

Resultaten en conclusies

De methode werkt goed voor alle geteste pathogenen, en is een efficiënte en vooral snelle methode om resistentie te detecteren. Aanvullende methoden zijn nodig ter bevestiging, omdat vals-negatieve resultaten niet geheel kunnen worden uitgesloten. De winst van de methode zit vooral in de snelheid en de potentiële toepasbaarheid te velde.

Toepasbaarheid

De methode voor het detecteren van ciprofloxacineresistentie is toepasbaar in situaties waarin de bacteriesoort reeds geïdentificeerd is. Direct daarna kan worden vastgesteld of resistentie aanwezig is. Indien dit het geval blijkt kan kostbare tijd gewonnen worden, en kunnen mogelijk zelfs levens gered worden, door een ander

Organisatie en financiering

In opdracht van het Ministerie van Defensie werd onderzoek gedaan naar methoden voor het aantonen van eventueel aanwezige resistentie-eigenschappen in bacteriën. Het onderzoek is uitgevoerd in de business unit 'Biologische en Chemische Bescherming' van TNO Defensie en Veiligheid. Het onderzoek is gefinancierd in het kader van programma V013 en hoort bij resultaatnummer 807b.

Probleemstelling

Wanneer resistentie vroegtijdig herkend wordt kan een meer effectieve therapie worden toegepast, en kunnen mogelijk levens worden gered. Het hier beschreven onderzoek bouwt voort op hetgeen beschreven is in TNO-rapport DV2 2005-A165, en beschrijft uitbreiding van de analysemethode.

Snelle detectie van ciprofloxacineresistentie – deel II

antibioticum toe te passen. De methode is breed toepasbaar, waarschijnlijk voor alle bacteriesoorten. De methode is in de huidige vorm in bijvoorbeeld een veldlaboratorium toepasbaar, mits daarvoor geschikte apparatuur (realtime PCR) aanwezig is. Realtime PCR is tevens zeer geschikt voor identificatie van pathogenen.

Vervolgafspraken

In programma V502 zal de ontworpen methode verder worden verbeterd en geschikt worden gemaakt voor een groter aantal bacteriesoorten.

PROGRAMMA	PROJECT
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Programmaleider Dr. M.S. Nieuwenhuizen, TNO Defensie en Veiligheid	Projectleider ir. M.P. Broekhuijsen, TNO Defensie en Veiligheid
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Summary

Background and goal

By order of the Dutch Ministry of Defence, a study was performed to develop or improve methods for the detection of antibiotic resistance in bacteria. When resistance is detected timely, a more successful therapy can be adopted, and lives might be saved. The current study builds on an earlier one that is described in TNO report DV2 2005-A165. The study was performed in the Business Unit 'Biological and Chemical Protection' of TNO Defence, Security and Safety. The study was financed under the program 'Protection against NBC weapons'.

Experimental study

The previously developed method was adjusted for and validated with several additional pathogens, including *Brucella melitensis* (causative agent of Brucellosis), *Yersinia pestis* (causative agent of Plague) and *Staphylococcus aureus* (including MRSA strains).

Results and conclusions

The method works well on all tested pathogens, and is an efficient and fast method for detecting ciprofloxacin resistance. Additional methods are necessary for confirmation, since false-negatives cannot be excluded entirely. The value of the method lies in speed and applicability in the field.

Applicability

The method for detecting ciprofloxacin resistance can be applied directly after the bacterial species has been identified, and within two hours after that. If resistance is detected, valuable time can be saved, or even lives, by applying another antibiotic. The method is applicable in field laboratories, provided real-time PCR equipment is present.

Further study

The method will be further improved, and extended to other relevant bacterial species.

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Abbreviations

ATCC	American Type Culture Collection
bp	base pair(s)
DNA	Deoxyribo-Nucleic Acid
dNTP	deoxy-Nucleoside-Tri-Phosphate
EtBr	Ethidium bromide
FOI	Swedish Defence Research Agency
FRET	Fluorescence Resonance Energy Transfer
grlA	topoisomerase IV, subunit A (or parC)
gyrA	topoisomerase II, subunit A (or gyrase A gene)
LB	Luria broth
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
RIVM	National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu)
TSA	Trypticase soy agar
QRDR	Quinolone resistance determining region

1 Introduction

1.1 Goal and approach of the project

The goal of the project is to investigate, develop, improve, or implement methods for the detection of antibiotic resistance in bacteria.

Most currently used methods for screening antibiotic resistance in a strain rely on culturing in the presence of antibiotics (Figure 1). Several commercial systems exist for this purpose. These methods were not explored or evaluated. The aim was to develop DNA-based methods, which are inherently faster than conventional methods and more suitable for field use.

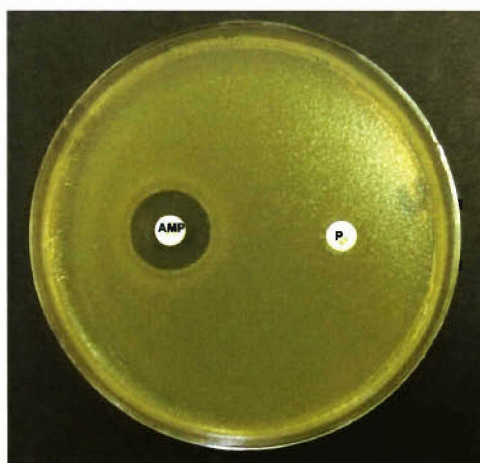


Figure 1 Example of antibiotic resistance testing using culture media. *Escherichia coli* bacteria are cultured as a layer on an agar plate. The susceptibility of the bacteria towards certain antibiotics is shown by applying a drop of antibiotic on the agar plate before culturing, resulting in a circular clear region (AMP) where bacteria will not survive. Resistant bacteria will multiply regardless of the presence of antibiotic (P). AMP: Ampicillin, P: penicillin.

The study is primarily directed towards operationally relevant antibiotics and strains, i.e. antibiotics that are commonly used for military personnel, and bacterial species that are considered to be biowarfare agents, or that are likely to be encountered by military forces during operations.

1.2 Background

When encountering bacterial infections in patients, or potentially pathogenic bacteria in environmental samples, the immediate need is to identify the bacteria. Once knowing the identity of the bacteria, one can prepare appropriate medical countermeasures. The immediately following concern is whether the identified bacteria carry any antibiotic resistance. Most bacterial infections can be treated effectively using antibiotic compounds, provided they are in sufficient stock and administered timely (early in the infection process). However, different bacterial species can vary in their susceptibility towards different antibiotic compounds. Even worse, many bacterial strains have developed resistance against specific antibiotics. Nowadays, many antibiotic compounds are known, both naturally occurring and of synthetic origin, which fall into

several classes, based on mechanism and action. Resistance against all known antibiotics has been described.

1.3 Previous work

In a previous report (Broekhuijsen and Van Dijk 2005a), general background information concerning the discovery of antibiotics, the development of resistance, and the mechanism of action and resistance have been described. In that report, experiments were described to detect antibiotic resistance associated with two types of resistance genes: acquired resistance genes and mutated genes that cause ciprofloxacin resistance. Experiments were then focused on the Quinolone Resistance Determining Region (QRDR) of so-called DNA topoisomerases (Broekhuijsen and Van Dijk 2005a), in particular the *gyrA* and *grlA* genes.

In another report (Broekhuijsen and Van Dijk 2005b), experiments were described to improve the method for detecting ciprofloxacin resistance, using real-time PCR. The improved method uses the so-called mutation assay of the LightCycler equipment, where hybridization probes are used in combination with ordinary PCR primers (Broekhuijsen and Boomaars 2001). This improved method, named QRDR mutation assay, was tested on *Serratia marcescens* and a non-virulent strain of *Bacillus anthracis*. To validate the method, ciprofloxacin resistant isolates of both strains were induced and tested.

1.4 Availability of resistant strains

In a previous report (Broekhuijsen and Van Dijk 2005b), the importance of having ciprofloxacin resistant strains was emphasized, because only then assays for detecting resistance can be truly validated. It was shown that induction of ciprofloxacin resistance is possible, and that the resistant isolates had mutations in the expected sequences. Preferably however, 'natural' resistant strains should be collected and tested, to verify if the designed assay is suitable for strains that might be encountered in real life. This was done by using a small collection of ciprofloxacin resistant MRSA strains. Of 6 resistant strains, 5 had a mutation in the expected region. Two different mutations were observed, but all at position 84 (amino acid numbering) in the QRDR of the *gyrA* gene.

The results obtained with the induced resistance in *Serratia marcescens* and *Bacillus anthracis*, and the 'natural' resistant strains of MRSA, together seemed to give enough confidence in the method. Therefore, no additional attempts were done in this study to induce resistance. Nor were new resistant isolates obtained from other sources.

1.5 Goal of the present study

The present study, described in this report, aims at extending the method for detecting ciprofloxacin resistance to additional pathogens, using the QRDR mutation assay described before (Broekhuijsen and Van Dijk 2005b). The method is tested on *Brucella melitensis*, *Yersinia pestis* and *Staphylococcus aureus*.

2 Materials and methods

2.1 Bioinformatics

DNA sequences were searched in GenBank on the NCBI website. Specific sequences of interest were downloaded and analyzed using the software package DNAMAN version 5.2.9 (Lynnon Biosoft, Canada). Primers were designed using the primer-design tool in DNAMAN, with 60+/-2 °C as preferred annealing temperature. DNA and protein sequences were aligned using the Multiple Sequence Alignment tool in DNAMAN with the default parameters. DNA sequences were submitted to GenBank using the online tool BankIt on the NCBI website.

2.2 Bacterial strains and DNA preparations

The strains or DNA preparations listed in Table 1 were used in this study.

Table 1 Strains or DNA preparations used in this study. 'TNO-code' refers to the collection at TNO Defence, Security and Safety, location Rijswijk. 'Other codes' refers to codes used by other organizations.

Species	TNO-code	Other codes	Comment
<i>Brucella abortus</i>	BM208	544, ATCC23448	DNA prep
<i>Brucella canis</i>	BM224	RM6/66, ATCC23365	DNA prep
<i>Brucella melitensis</i>	BM215	16M, ATCC23456	DNA prep
<i>Brucella suis</i>	BM218	1330, ATCC23444	DNA prep
<i>Yersinia pestis</i>	BM043	KIM5	Cell-lysate
<i>Yersinia enterocolitica</i>	BM112	ATCC29913	Cell-lysate
<i>Yersinia pseudotuberculosis</i>	BM115	ATCC29833	Cell-lysate
<i>Staphylococcus aureus</i>	BM187	ATCC29213	
<i>Staphylococcus aureus</i>	BM292	02-00855	MRSA
<i>Staphylococcus aureus</i>	BM293	02-00664	MRSA, cipro-resistant
<i>Staphylococcus aureus</i>	BM294	02-00279	MRSA, cipro-resistant
<i>Staphylococcus aureus</i>	BM295	02-00578	MRSA, cipro-resistant
<i>Staphylococcus aureus</i>	BM296	02-00880	MRSA, cipro-resistant
<i>Staphylococcus aureus</i>	BM297	01-01177	MRSA, cipro-resistant
<i>Staphylococcus aureus</i>	BM298	02-00679	MRSA, cipro-resistant

The *Brucella* DNA preparations were kindly provided by dr. G. Vergnaud, Université Paris-Sud, Orsay, France. The *Y. pestis* KIM5 lysate was kindly provided by dr. Mats Forsman from FOI, Sweden. Live *Y. pseudotuberculosis* and *Y. enterocolitica* were obtained from ATCC. *S. aureus* strain BM187 was kindly provided by a local hospital. All other *S. aureus* strains were kindly provided by dr. Wieger Homan from the Dutch National Institute for Public Health and the Environment (RIVM) in Bilthoven, The Netherlands. All live strains were cultured in LB liquid medium or on TSA plates, at 37 °C.

Thermolysates were prepared by heating 1 ml of overnight culture in LB at 95 °C for 20 minutes. Thermolysates were analyzed for any residual presence of live agent by plating out on TSA and culturing for 5 days at 37 °C. Inactivated thermolysates were used for conventional as well as real-time PCR, after 1:100 dilution in water.

2.3 Conventional PCR and gel electrophoresis

Conventional PCR was performed using an MJ Research PTC-200 thermocycler, standard PCR tubes of 0.5 ml volume, with a reaction mix volume of 50 μ l. Taq-polymerase and dNTP-mix was used from Roche Diagnostics. End concentrations in the PCR reaction mixture were 2.5 units Taq-polymerase, 1.5 mM $MgCl_2$, 0.2 μ M primers, 1 mM dNTP. A standard program was used with the following parameters:

- 1 5 min. 92 °C
- 2 30 sec. 92 °C
- 3 30 sec. 60 °C
- 4 30 sec. 72 °C
- 5 goto 2, 34 times
- 6 5 min. 72 °C
- 7 15 °C for ever
- 8 end.

After PCR, a 10 μ l sample was analyzed on a standard 1.2% agarose gel in 0.5x TBE buffer (0.89 M Tris borate, 0.02 M EDTA, pH 8.3). Loading buffer was used according to Sambrook et al (1989). Electrophoresis was performed during 45 minutes at 100 mA. Gels were visualized using EtBr. Molecular weight markers used was M-9 (digest of DNA from Φ X174 with HinfI, obtained from Eurogentec), containing DNA fragments of 726, 713, 553, 500, 427, 417, 413, 311, 249, 200, 151, 140, 118, 100, 82, 66, 48, 42, 40, 24 bp.

2.4 Real-time PCR

The QRDR mutation assay was performed as a real-time PCR assay, using the LightCycler (Roche Diagnostics), with a reaction volume of 20 μ l. Reagents were used from Roche Diagnostics, e.g. the DNA Master SYBR Green I kit and the DNA Master HybProbe kit. Hybridization probes were obtained from TibMolbiol in Berlin, Germany. The QRDR mutation assay was performed essentially according to literature (Lindler et al. 2001). except that primer and probe sequences were adapted to the specific sequence of the *gyrA* gene, and minor modifications of the program parameters.

PCR reaction mix for the QRDR mutation assay:

- 2.0 μ l forward/reverse primers (each 10 μ M)
- 2.0 μ l probes (each 4 μ M)
- 2.0 μ l DNA
- 3.2 μ l $MgCl_2$ (25 mM)
- 2.0 μ l Hot start reaction mix (with 10 mM $MgCl_2$)
- 8.8 μ l H_2O

End concentration $MgCl_2$ = 5 mM

The PCR reaction conditions for the QRDR mutation assay are described in Table 2.

Table 2 Program parameters used for the QRDR mutation assay.

Segment number	Temperature Target (°C)	Hold time (sec)	Slope (°C/sec)	Acquisition mode
Program: Denaturation				Cycles:1
1	95	600	20	None
Program: Amplification				Cycles:40
1	95	10	20	None
2	55-58 [†]	15	20	Single
3	72	15	20	None
Program: Amplification2				Cycles:1
1	72	30	20	None
Program: Melting				Cycles:1
1	95	0	20	None
2	40	30	20	None
3	95	0	0.1	Continuous
Program: Cooling				Cycles:1
1	40	30	20	None

[†] 55 °C annealing was used for *S. aureus*, 58 °C annealing was used for all other species.

2.5 Design of hybridization probes

Hybridization probes were designed by comparing the probes used before (Broekhuijsen and Van Dijk 2005b) with the target sequence, and adapted accordingly. If possible, probes were designed according to guidelines of TibMolbiol (Berlin, GE). These guidelines (below) are copied from their text. T_m is the melting temperature of the hybridized probe (probe-template double helix).

Avoid these probe sequences....

- Repetitive or monotonous sequences;
- Self-complementary sequences;
- Cluster of Gs and Cs at either end of probe;
- Probe extremely rich in purines (G and A);
- Sequences that can hybridize with the 3' termini of PCR primers.

Guideline for probe T_m's

- T_m's of the probes should be at least 5-10 °C above T_m's of the primers. Avoid T_m's >10-20 °C higher than primer T_m;
- For mutation analysis, the T_m of the sensor probe should be distinctly less than the T_m of the anchor probe;
- Sensor probe covers the predicted site of the mutation while the anchor probe produces the fluorescent signal.

2.6 Primers and probes

Properties of primers and probes used in this study are listed in Table 3. Primers and probes for the *gyrA* gene of *Yersinia* were taken from literature (Lindler et al. 2001). For the *gyrA* gene of *B. melitensis* and *S. aureus*, primers and probes were designed in this study, and are confidential. Primers GyrA-F01 and GyrA-R03 (Broekhuijsen and Van Dijk 2005a) were used for sequencing of *Y. enterocolitica*.

Table 3 Primer and probe data. The name of the primer or probe reflects the target gene (*gyrA*). Primers are either forward (F) or reverse (R). Probes are designated with P. n.a.: not applicable. Target organisms: Bru = *Brucella*, Yer *Yersinia*, Sau = *S. aureus*.

Name	Sequence (5' - 3') ¹	Label (5')	Label (3')	Target organism
GyrA-F01	TC(CG)TATCTGGA(CT)TATGCGATGTC	n.a.	n.a.	n.a.
GyrA-R03	CGAATTTCGGTATAACGCAT	n.a.	n.a.	n.a.
GyrA-F12	confidential	n.a.	n.a.	Bru
GyrA-R12	confidential	n.a.	n.a.	Bru
GyrA-F14	confidential	n.a.	n.a.	Bru
GyrA-R14	confidential	n.a.	n.a.	Bru
GyrA-F15	GATTATGCGATGTCCGTTATTGTC	n.a.	n.a.	Yer
GyrA-R15	GAAGTTACCCTGCCCATCCAC	n.a.	n.a.	Yer
GyrA-F16	confidential	n.a.	n.a.	Sau
GyrA-R16	confidential	n.a.	n.a.	Sau
GyrA-P09	confidential	n.a.	Fluorescein	Bru
GyrA-P10	confidential	Red640	n.a.	Bru
GyrA-P07	GCATGGTGACAGCGCGGTC	n.a.	Fluorescein	Yer
GyrA-P08	ACGACACTATCGTGCGTATGGCCCA	Red640	n.a.	Yer
GyrA-P11	confidential	n.a.	Fluorescein	Sau
GyrA-P12	confidential	Red640	n.a.	Sau

¹ Nucleotides within brackets represent degenerate positions, with a mix of both nucleotides at that position, e.g. (CG) denotes C or G at a single position.

2.7 DNA sequencing

DNA sequencing was performed using amplicons as template and primers the same as for amplifying the DNA. Amplicons were purified with the GeneClean Spin kit, according to the manufacturers instructions. Purified amplicons and PCR primers were sent to the company Baseclear in Leiden for sequencing, using their standard procedures. Resulting DNA sequences were received from Baseclear by email. The sequence of *gyrA* of *Y. enterocolitica* was determined using primers GyrA-F01 and GyrA-R03.

3 Results

This report describes the development and validation of additional QRDR mutation assays, similar to those developed before (TNO report DV2 2005-A165). The newly developed QRDR mutation assays target the *gyrA* gene of a Gram-negative pathogen (*Brucella*) and a Gram-positive pathogen (*S. aureus*). In addition, the published QRDR mutation assay for *Yersinia pestis* (Lindler et al. 2001) was validated by testing it on two other species of *Yersinia*.

To design new QRDR mutation assays, the DNA sequence of the QRDR must be known, or elucidated. The availability of *gyrA* QRDR sequences in GenBank is described. Next, the result of the analysis of the DNA sequence of the QRDR of the *gyrA* gene of *Y. enterocolitica* is described. This sequence was particularly useful in validating the QRDR mutation assay for *Yersinia*.

Next, the design strategy for the QRDR mutation assay is described. Finally, the QRDR mutation assays for *Brucella*, *Yersinia* and *S. aureus* are described.

3.1 Available *gyrA* QRDR sequences

The sequences of the *gyrA* genes of *Brucella melitensis*, *B. suis* and *B. abortus* can be found in GenBank. These were downloaded and the first 420 bp of the gene were used for further analysis and design. There were no other *Brucella gyrA* sequences available, e.g. of *B. canis*. The DNA sequences of the first 420 bp of the known *Brucella gyrA* sequences are identical. It is likely that other *Brucella gyrA* gene sequences are also identical, because *Brucella* is genetically very homogeneous, and often considered a single species with different biovars.

The sequence of the *gyrA* gene of *Yersinia pestis* is also available in GenBank. The sequence of the *gyrA* gene of *Y. pseudotuberculosis* is only partially available, but includes the entire QRDR and lacks only the first 21 bp of the *gyrA* gene. The available part is identical to the *gyrA* sequence of *Y. pestis*, which is not surprising because *Y. pestis* and *Y. pseudotuberculosis* are considered to be close relatives. The equivalent sequence of *Y. enterocolitica* is also partially available in GenBank (accession number AY064398), but is only 171 bp long, which is not sufficient. This sequence seemed to be of value, and was therefore determined in this study (below).

The sequence of the *gyrA* gene of *S. aureus* has been determined before, and several mutations were found in the QRDR of some of the MRSA strains of *S. aureus* (TNO report DV2 2005-A165).

3.2 QRDR sequence of *gyrA* of *Y. enterocolitica*

The sequence of the QRDR of the *gyrA* gene of *Y. enterocolitica* was determined by first amplifying the genomic region using primers GyrA-F01 and GyrA-R03. The amplicon was purified and then sequenced using the same primers. The resulting sequence covered the entire amplicon (320 bp). The first 57 bp of the *gyrA* gene of *Y. enterocolitica* are not represented in this sequence, but the relevant region (the QRDR) is included. The sequence of 320 bp (Figure 2) was submitted to GenBank (Accession no. DQ317602).

```

58      TCGTATCTGGATTATGCGATGTCCGTTATTGTCGGACGTGCGTTACCAGATGTCCGGAT
20      S Y L D Y A M S V I V G R A L P D V R D

118     GGACTGAAACCGGTGCACCGTCGCGTACTGTTTTCGATGAATGTACTGGGTAATGACTGG
40      G L K P V H R R V L F A M N V L G N D W

178     AATAAGCCATACAAAAATCGGCCCGTGTAGTCGGGGACGTTATCGGTAAATATCACCCG
60      N K P Y K K S A R V V G D V I G K Y H P

238     CATGGTGACAGCGCGGTCTACGACCAATGTGCGTATGGCCAGCCGTTCTCACTGCGC
80      H G D S A V Y D T I V R M A Q P F S L R

298     TATATGCTGGTGGATGGGCAGGGCAACTTCGGTTCGGTGATGGCGACTCCGCCGCGCG
100     Y M L V D G Q G N F G S V D G D S A A A

358     ATGCGTTATACCGAAATTCG
120     M R Y T E I R

```

Figure 2 Genomic and derived amino acid (one letter code) sequences of the QRDR of the *gyrA* gene of *Y. enterocolitica*. Numbering is shown according to the sequences of *Y. pestis*. Base changes compared to *Y. pestis* are highlighted.

Eleven single base changes (Figure 2) were found in the *Y. enterocolitica* QRDR sequence of 320 bp (97% homology) when comparing with the equivalent sequences of *Y. pestis* and *Y. pseudotuberculosis* (the two of which are identical). The amino acid sequences corresponding to the 320 bp are identical in all three *Yersinia* species.

Additionally, four single base changes were found when comparing to another partial sequence of the *gyrA* gene of *Y. enterocolitica* (171 bp) published in GenBank (accession number AY064398), but the amino acid sequence again is identical.

3.3 Design strategy of QRDR mutation assay

In Gram-negative bacteria, the QRDR of the *gyrA* gene is the obvious target for development of a real-time PCR assay for detection of ciprofloxacin resistance (Broekhuijsen and Van Dijk 2005a and 2005b). If possible, common assays for different bacteria are preferred, i.e. one assay suitable for several species. This can only be achieved if the target DNA sequences shows sufficient homology.

The homology of the *gyrA* genes of the *Brucella* and *Yersinia* species used in this study was analyzed by multiple sequence alignment, shown in Figure 3. As shown, the DNA sequences (of the first 420 bases of the *gyrA* gene) of the three *Brucella* species are identical. *Y. enterocolitica* is 97% homologous to *Y. pestis* and *Y. pseudotuberculosis* (the two of which are identical). This means that designing a single assay for the *Yersinia* species should be easy. In contrast, the *Brucella* and *Yersinia* groups are only 66% homologous. A single assay for these two groups is therefore not feasible.

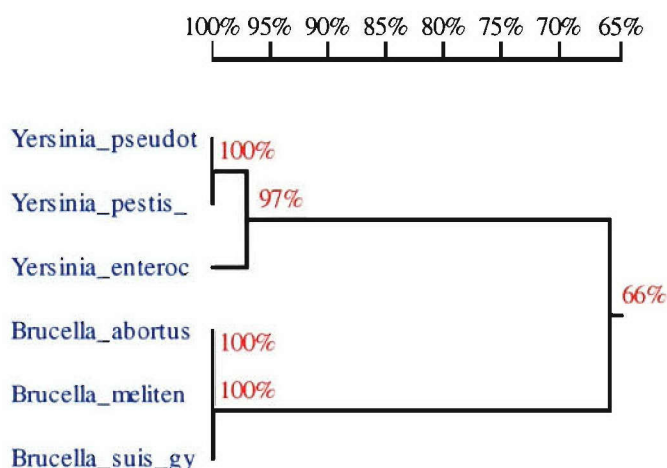


Figure 3 Homology tree of the first 420 bases of the *gyrA* gene of *Y. pseudotuberculosis*, *Y. pestis*, *Y. enterocolitica*, *B. abortus*, *B. melitensis*, and *B. suis*.

In Gram-positive bacteria, contrary to Gram-negative bacteria, the *grlA* gene also seems to be of importance (Broekhuijsen and Van Dijk 2005a). However, in both Gram-positive bacteria investigated so far (*B. anthracis* and *S. aureus*, Broekhuijsen and Van Dijk 2005b), ciprofloxacin resistant strains or isolates mostly had mutations in the expected region (QRDR) in the *gyrA* gene. Some strains or isolates also had a mutation in the QRDR of the *grlA* gene. There was one exception in a resistant strain of *S. aureus*, where no mutation in the QRDR was found, neither in the *gyrA* gene nor in the *grlA* gene.

Most importantly, all strains or isolates that had a mutation in the QRDR of the *grlA* gene, also had a mutation in the QRDR of the *gyrA* gene. The QRDR of the *gyrA* gene thus seems to be the most relevant target, and designing an additional assay for the QRDR of the *grlA* gene seems to be of limited additional value. Therefore, it was decided to focus on the QRDR of the *gyrA* gene, in both Gram-negative and Gram-positive species.

3.4 QRDR mutation assay for *Brucella*

All QRDR sequences of *gyrA* genes of *Brucella* in GenBank are identical. From this sequence, several primers and probes were designed, not all of which are described here. Primers GyrA-F12, -R12, -F14 and -R14 produced sufficient amplicon, and probes GyrA-P09 and -P10 showed good results in combination with all these primers. The relevant area of the QRDR of the *gyrA* gene of *Brucella* is shown in Figure 4, both the genomic and amino acid sequences.

Bru ATGGGTAAGTATCACCCGCATGGCGATGCTTCGATCTATGATGCCCTCGTGCGT
M G K Y H P H G D A S I Y D A L V R
78 81 83 87 95

Figure 4 Sequence of the relevant area of the QRDR of the *gyrA* gene of *Brucella* (Bru). The genomic and amino acid (one letter code) sequences are shown, together with the amino acid numbering.

For the *Brucella* strains, two primer pairs (GyrA-F12 and GyrA-R12; GyrA-F14 and GyrA-R14) worked equally well, and both are presented here. Both were used in combination with the same two probes (GyrA-P09 and GyrA-P10). Figures 5 and 6 show the results with these two primer pairs.

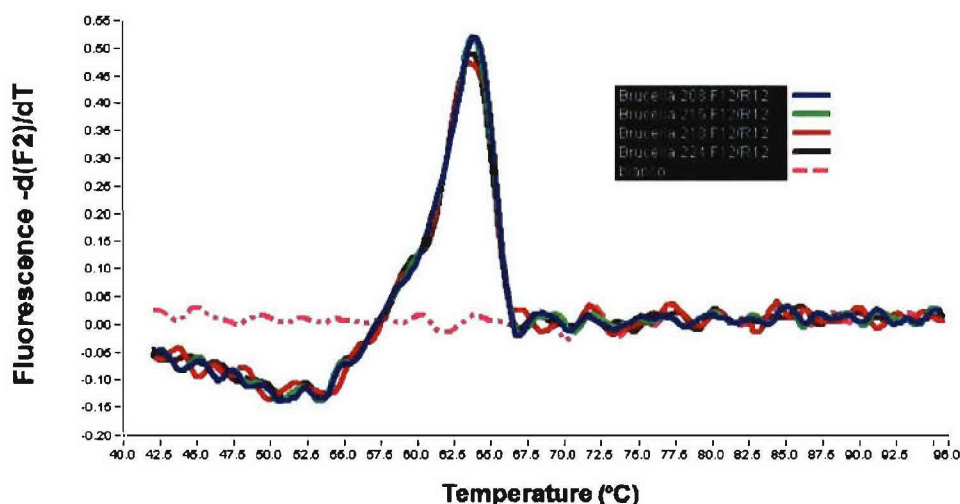


Figure 5 QRDR mutation assay for *Brucella*, using primers GyrA-F12 and GyrA-R12. The numbers 208, 215, 218 and 224 refer to the strain numbers (Table 1 and text).

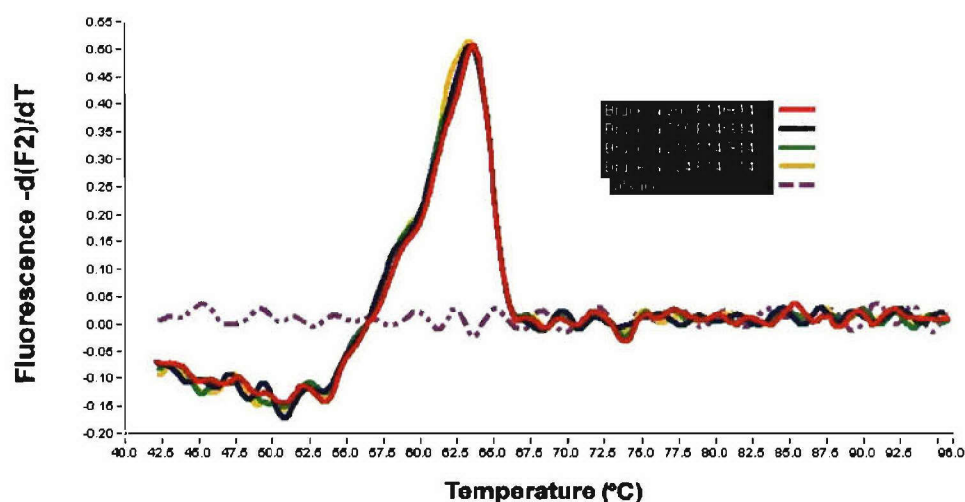


Figure 6 QRDR mutation assay for *Brucella*, using primers GyrA-F14 and GyrA-R14. The numbers 208, 215, 218 and 224 refer to the strain numbers (Table 1 and text).

The QRDR mutation assay was tested on four strains of *Brucella*, BM208 (*B. abortus*), BM215 (*B. melitensis*), BM218 (*B. suis*) and BM224 (*B. canis*). Since all sequences are identical, no difference in melting point is visible. In fact, the identical melting points demonstrate that the sequence at the probe locations in *B. canis* (which was unknown) is identical as well. The observed melting point is 63.5 °C.

3.5 QRDR mutation assay for *Yersinia*

For *Yersinia*, there are two different genomic sequences of the QRDR of the *gyrA* gene. The primers and probes used are taken from literature (Lindler et al. 2001). These primers and probes are based on the sequence of *Y. pestis*, which is identical to that of *Y. pseudotuberculosis*. The equivalent sequence of *Y. enterocolitica*, however, differs. This is indicated in Figure 7.

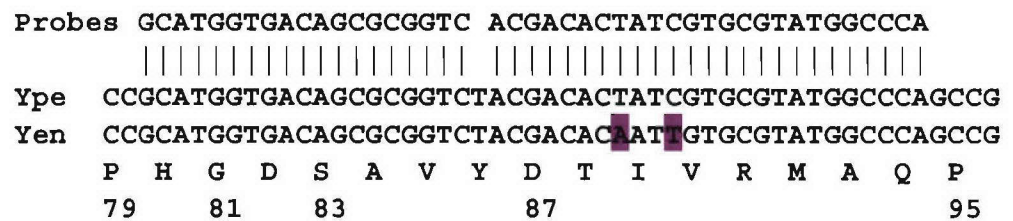


Figure 7 Sequences of the relevant area of the QRDR of the *gyrA* gene of *Y. pestis* (Ype) and *Y. enterocolitica* (Yen). Probes GyrA-P07 and GyrA-P08 are shown in the top line. The genomic and amino acid (one letter code) sequences are also shown, together with the amino acid numbering. Base changes in the *Y. enterocolitica* sequence are highlighted. The amino acid sequence is the same for both species.

All three species were tested with the primers and probes. As expected, *Y. enterocolitica* showed a different melting point than the other two (Figure 8), because there are two single base changes in the area where the probes hybridize. The melting point for *Y. pestis* and *Y. pseudotuberculosis* is 71 °C and for *Y. enterocolitica* 63 °C (Figure 8).

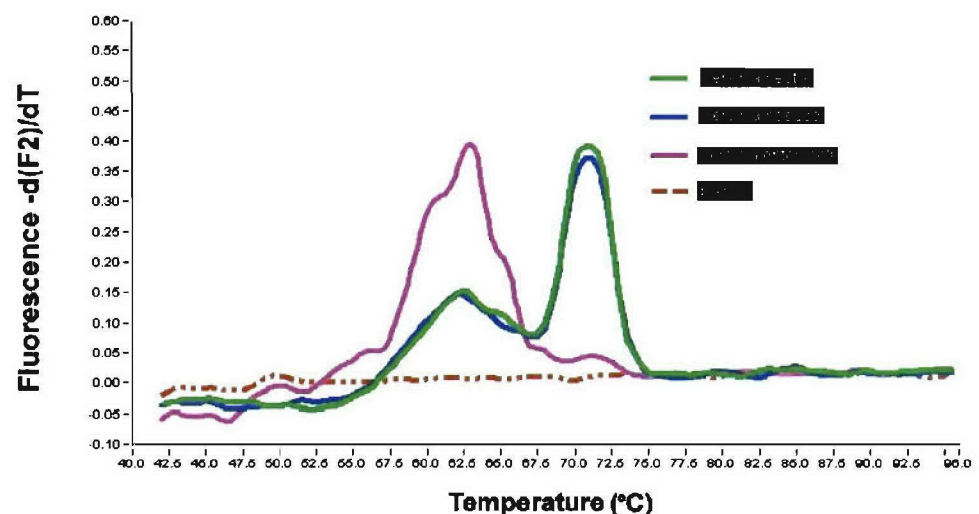


Figure 8 QRDR mutation assay for *Yersinia*. The right peak is from *Y. pestis* and *Y. pseudotuberculosis*, on which the probe sequences are based. The left peak is from *Y. enterocolitica*.

3.6 QRDR mutation assay for *S. aureus*

The set of *S. aureus* strains used in this study have been described previously, including the DNA sequence of the QRDR of the *gyrA* gene of all these strains (Broekhuijsen and Van Dijk 2005b). The relevant area in the QRDR of the *gyrA* gene is shown in Figure 9. Four different DNA sequences were found. The wildtype sequence is represented by strain BM187, and is the same for strains BM292 and BM297. Strains BM293, BM296 and BM298 have a single base change resulting in a Serine (S) to Leucine (L) change at amino acid position 84. Strain BM294 also has a single base change, but a different one, resulting in a Serine (S) to Alanine (A) change at amino acid position 84. Finally, strain BM295 has two single base changes, one of which results in a Serine (S) to Leucine (L) change at amino acid position 84 (Figure 9).

BM187	CCTCATGGTGACTCATCTATTTATGAAGCAATGGTACGTATGGCTCAAGATTTTCAGT
	P H G D S S I Y E A M V R M A Q D F S
BM293	CCTCATGGTGACTTATCTATTTATGAAGCAATGGTACGTATGGCTCAAGATTTTCAGT
	P H G D L S I Y E A M V R M A Q D F S
BM294	CCTCATGGTGACTCATCTATTTATGAAGCAATGGTACGTATGGCTCAAGATTTTCAGT
	P H G D A S I Y E A M V R M A Q D F S
BM295	CCTCATGGTGACTTATCTATTTATGAAGCAATGGTACGTATGGCTCAAGATTTTCAGT
	P H G D L S I Y E A M V R M A Q D F S
	80 84 98

Figure 9 Sequences and mutations in the QRDR of the *gyrA* gene of several *S. aureus* strains. BM187 represents the wildtype sequence, on which probes GyrA-P11 and GyrA-P12 are based. Three deviating genomic sequences were found in the other *S. aureus* strains, represented by BM293, BM294 and BM295. For each strain, the genomic and amino acid (one letter code) sequences are shown. Mutations are highlighted. The amino-acid numbering is shown in the bottom line.

All eight available strains of *S. aureus* were tested with the designed primers and probes. The result is shown in Figure 10. As expected, the strains with a wildtype sequence (BM187, BM292 and BM297) have the highest melting point (61-62 °C). The strains with one single base change (BM293, BM294, BM296 and BM298) have an intermediate melting point (57-58 °C). Strain BM295 with two single base changes shows the lowest melting point (54 °C).

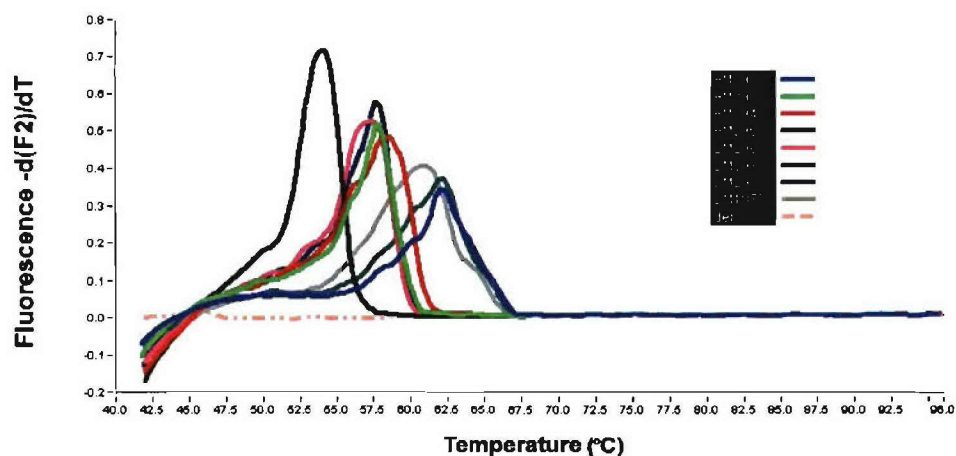


Figure 10 QRDR mutation assay for the *gyrA* gene of *S. aureus*. The three peaks on the right are from strains with a wildtype sequence (BM187, BM292 and BM297). The middle peaks are from strains having one single base mutation and a mutated amino acid at position 84 (BM293, BM294, BM296 and BM298). The left peak is from BM295, which has two single base mutations.

4 Discussion and conclusions

4.1 QRDR sequences

It is necessary to know the DNA sequence in order to design a QRDR mutation assay. However, if the sequence is not available in GenBank, it is not always so difficult to obtain it. Because *gyrA* is an evolutionary conserved gene, the sequence is mostly homologous to other, known *gyrA* genes. This means that primers often fit and an amplicon of another species can be obtained, suitable for sequencing. The sequencing of the QRDR of the *gyrA* gene of *Y. enterocolitica*, described in this report, illustrates this. Even if the primers do not fit perfectly, an amplicon is sometimes obtained.

The *gyrA* QRDR sequences of the *Brucella* species are identical. This is expected because of the genetic homogeneity of the genus *Brucella*. In fact, the different 'species' of the genus *Brucella* are taxonomically considered different biovars of one species (*B. melitensis*), e.g. *B. melitensis* biovar *abortus*, according to ICSB, Subcommittee on the Taxonomy of *Brucella* 'Report of the meeting, 5 September 1986, Manchester, England.' Int. J. Syst. Bacteriol. (1988) 38:450-452. (Note: *Brucella* is a monotypic genus (*B. melitensis*). However, the nomen species *B. abortus*, *B. canis*, *B. neotomae*, *B. ovis*, and *B. suis* can be retained for nontaxonomic purposes to avoid confusion.).

The *gyrA* QRDR sequences of *Y. pestis* and *Y. pseudotuberculosis* are also identical, as expected, being closely related. However, the equivalent sequence of *Y. enterocolitica* revealed 11 single base changes in 320 bp (96.6 % homologous), and this species is obviously more distantly related to the other *Yersinia* species. A striking difference between the sequenced QRDR of *Y. enterocolitica* in this report and one that was available in GenBank (accession number AY064398) was observed: 4 single base changes in 171 bp, meaning 97.7 % homologous. This is a rather large difference for a conserved gene in the same species. Obviously, these two strains are quite different. It is unknown how heterogeneous *Y. enterocolitica* as a species is. Although the DNA sequences might differ, the derived amino acid sequences are all identical for the *Yersinia* species, again reflecting the conserved nature of the *gyrA* gene.

The *gyrA* QRDR sequences of the *S. aureus* strains show several base changes when compared to the wildtype sequence, most of which translate into amino acid changes. This is quite heterogeneous within a species, but maybe not surprising, because this might be the result of genetic pressure, perhaps the result of exposure to ciprofloxacin or a related compound.

4.2 Designing QRDR mutation assays

Designing and testing QRDR mutation assays proved successful for all strains tested here. However, the design of the QRDR mutation assay for *Brucella* proved more difficult than the others. The probe pair described here was the third probe pair tested, whereas the first two probe pairs did not function. Attempts were made to examine the sequence in the *Brucella gyrA* QRDR, but no obvious property could explain the difficulty of designing effective probes. The GC-content (a possible criterion) is not apparent: the percentage of GC in functional probes varies from 39.3 %GC for probe

GyrA-P11 to 68.4 %GC for probe GyrA-P07, a wide range. The *Brucella* probes GyrA-P09 and GyrA-P10 are in the middle range of these values, with 59.1 and 50.0 %GC, respectively.

Worldwide, designing hybridization probes for real-time PCR is often outsourced to the company TibMolbiol, with the argument that specialized knowledge and experience is required. They also claim a high success rate. In the case of designing a QRDR mutation assay, the room for variation is limited. The probes should always cover the region where mutations are expected. Therefore, probes were designed in-house. The criteria for designing probes, as communicated by TibMolbiol (Materials and methods), were used as far as possible. It is questionable whether all criteria are important. At least one criterion seems not to be essential: The criterion that states that the sensor probe (the left one) should cover the mutation site and the anchor probe (the right one) should not. This is contradicted by the clear distinction of the melting peak of *Y. enterocolitica*, which has two single base mutations in the anchor probe area. The resistant strains described in literature (Lindler et al. 2001) have mutations in the sensor probe area, using the same primers and probes. Obviously, there is no preference.

The two probes used in the QRDR mutation assay hybridize closely together, with only one nucleotide distance. This is common practice when hybridization probes are used in real-time PCR. The average length of the probes used so far is 23.5 bases, which means that an area of 47 bases is covered. Presumably, any mutation in this area would result in a change in melting point, with the possible exception of the first and last bases of the probes. Also, the single nucleotide between the two probes is not examined at all. This should be considered when designing QRDR mutation assays.

4.3 QRDR mutation assay for *Brucella*

Despite the difficulty in designing the assay for *Brucella* (see above), the final assay seems to work well. As shown, two alternative primer pairs worked equally well. However, the functionality of the assay could not be verified with a mutant strain. All available strains have an identical QRDR sequence, and no difference in melting point could be observed. Attempts will be made to find *Brucella* strains with mutations in the QRDR, although this might prove unlikely, as *Brucella* seems to be genetically very homogeneous. An alternative would be to induce ciprofloxacin resistance. This should only be done with a non-virulent strain, or possibly with a strain that is not a human pathogen.

4.4 QRDR mutation assay for *Yersinia*

The QRDR mutation assay for *Yersinia* was copied from literature (Lindler et al. 2001), where it was described for use on *Y. pestis*. In this study, the assay was also tested with two other species, *Y. pseudotuberculosis* and *Y. enterocolitica*. The last proved to be of additional value, because of base changes in the probe area. This resulted in a different melting point, demonstrating the functionality of the assay. Interestingly, the base changes in *Y. enterocolitica* are in the anchor probe area, as opposed to the base changes in resistant *Y. pestis* strains (Lindler et al. 2001), which are in the sensor probe area. This demonstrates that both probes are functional in scoring mutations.

4.5 QRDR mutation assay for *S. aureus*

This QRDR mutation assay was designed in-house, and proved successful. All mutations were clearly detected by a shift in melting point. The strain with two base changes (BM295) showed a further shift in melting point, demonstrating additional analytical value of the assay. Analyzing the results obtained with *S. aureus*, a general remark should be made. The QRDR mutation assay scores mutations in the DNA sequence, which is not synonymous with ciprofloxacin resistance. This is illustrated in two ways:

- 1 Strain BM295 has two single base changes, but only one reflects an amino acid change, responsible for resistance. The other base change is a silent mutation, which is detected by the mutation assay, but has no relevance for resistance.
- 2 Strain BM297 has no mutations in the QRDR of the *gyrA* gene, nor in the QRDR of the *grlA* gene (Broekhuijsen and Van Dijk 2005b). The melting point is indistinguishable from that of the wildtype strain. However, BM297 is ciprofloxacin resistant. In this case, a mutation in another gene must be responsible.

This illustrates that the QRDR mutation assay, although fast and efficient, is not always conclusive. Consideration should be given to the possibility of scoring false positives or false negatives.

4.6 QRDR mutation assays sofar

Sofar, QRDR mutation assays have been designed and validated for the following species and genes (Table 4).

Table 4 QRDR mutation assays developed and validated sofar in this project.

Species or genus	Gram-stain	Gene
<i>Serratia marcescens</i>	negative	<i>gyrA</i>
<i>Bacillus anthracis</i>	positive	<i>gyrA</i>
<i>Bacillus anthracis</i>	positive	<i>grlA</i>
<i>Brucella</i>	negative	<i>gyrA</i>
<i>Yersinia</i>	negative	<i>gyrA</i>
<i>Staphylococcus aureus</i>	positive	<i>gyrA</i>

For *S. marcescens* and *B. anthracis*, resistant isolates were induced, and were used to validate the method. For *S. aureus*, resistant strains were obtained from elsewhere, and used for validation. The assay for *Yersinia* was validated by using *Y. enterocolitica*, which has base changes compared to *Y. pestis*. The assay for *Brucella* could not be validated with mutant strains sofar.

4.7 Alternative method: DNA microarray

Recently, an alternative DNA-based method for detecting ciprofloxacin resistance was published (Zhou et al. 2004). The study used sensitive and resistant strains of *Neisseria gonorrhoeae* and analyzed the *gyrA* and *grlA* genes both with DNA sequence analysis and DNA microarray. The DNA microarray consisted of oligonucleotides aimed at detecting mutations in the relevant genes. Using 87 clinical specimens, they demonstrated that the DNA microarray detected all mutations that were revealed by DNA sequencing. They conclude that ' the oligonucleotide biochip technology has

potential utility for the rapid and reliable identification of point mutations in the drug resistance genes of *N. gonorrhoeae*.

The word 'rapid' in their statement should be taken relatively. Currently, DNA microarray analysis is much slower and more complex than real-time PCR. However, as technology moves on, DNA microarray might become an attractive alternative for detecting resistance, especially when it can be combined with identification of microorganisms (Broekhuijsen 2005, in press).

4.8 Operational issues

Operational issues have been discussed before (Broekhuijsen and Van Dijk 2005b). Arguments were given for the ease of designing, ease of use, use in a field environment, and other matters. The time required for the assay was estimated as 90 minutes, possibly shorter depending on skill and experience of operators. The success rate of the method was discussed and estimated as 80-90% or more. These conclusions and estimates have not changed since.

5 Further research and recommendations

5.1 QRDR mutation assay

Further research can be based on the results described in this report. The QRDR mutation assay is shown to be effective, and can be extended to other relevant species. It should be designed for the *gyrA* gene for all relevant species, and possibly also for the *griA* gene for Gram-positive species. Other species might include *Vibrio cholerae*, *Francisella tularensis*, *Burkholderia mallei*, *Coxiella burnetii* and *Escherichia coli* (O157).

Adaptation of the method for the *gyrB* gene and *griB* gene can be considered, but this should be preceded by DNA sequence analysis of these genes from highly resistant strains. It should only be done if mutations are found in these genes. If mutations are not found in these genes, this would strengthen the case for the *gyrA* gene as primary target.

5.2 Resistant strains

Resistant strains remain essential for validating the method. It would be desirable to have (or to induce) more resistant strains, of different species. 'Natural' resistant strains are to be preferred. Extra effort into obtaining resistant strains, although time consuming and often fruitless, should be considered.

5.3 DNA microarray

An alternative approach, to develop DNA microarray for detection of ciprofloxacin resistance (Zhou et al. 2004), can be considered to investigate. Zhou et al show that this is possible using an oligonucleotide microarray. A microarray approach is already being developed for identification of pathogens in another project for Defence (Broekhuijsen 2005, in press). It should be considered to mix these two topics, i.e. by incorporating specific oligonucleotides aimed at detecting ciprofloxacin (and perhaps other antibiotics) resistance on the microarray, together with oligonucleotides which are meant for identification of microorganisms. Together, this could be a very efficient way of analysis of unknown (clinical or environmental) samples.

5.4 Other matters

It is recommended to seek for exchange of information and data on this topic with other parties, e.g. foreign defence research. The Swedish Defence Research Agency FOI has expressed interest, and received a copy of an earlier report (Broekhuijsen and Van Dijk 2005a). If FOI continues this effort, exchange of information and complementarity of research will be effected.

If the method is considered to be implemented for operational use, the design of ready-to-use reagent mixtures can be considered, as well as testing the performance and stability of these.

Resistance against other antibiotics can be considered as another topic for future research.

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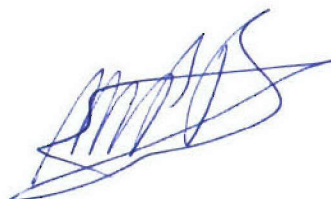
7 Signature

Rijswijk, January 2006

TNO Defence, Security and Safety

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Dr. M.S. Nieuwenhuizen
Head of Department

A handwritten signature in blue ink, appearing to be 'M.P. Broekhuijsen', written in a cursive style.

M.P. Broekhuijsen, M.Sc.
Author, projectleader

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W.C.M. van Dijk, B.Sc.
Author

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| 21/25 | TNO Defensie en Veiligheid, vestiging Rijswijk,
Business Unit BC Bescherming
Dr. N.D. Zegers, Dr. M. Polhuijs, Dr. D. van der Kleij, Dr. J. Kieboom,
Dr. F.J. Bikker |
| 26 | TNO Defensie en Veiligheid, vestiging Rijswijk,
Marketing en Communicatie, digitale versie via Archief |
| 27 | TNO Defensie en Veiligheid, vestiging Rijswijk,
afdeling Tekstverwerking (digitale versie) |

Onderstaande instanties/personen ontvangen het managementuittreksel en de distributielijst van het rapport.

- 4 ex. DMO/SC-DR&D
- 1 ex. DMO/ressort Zeesystemen
- 1 ex. DMO/ressort Landsystemen
- 1 ex. DMO/ressort Luchtsystemen
- 2 ex. BS/DS/DOBBP/SCOB
- 1 ex. MIVD/AAR/BMT
- 1 ex. TNO Defensie en Veiligheid, Algemeen Directeur,
ir. P.A.O.G. Korting
- 1 ex. TNO Defensie en Veiligheid, Directie
Directeur Operaties, ir. C. Eberwijn
- 1 ex. TNO Defensie en Veiligheid, Directie
Directeur Kennis, prof. dr. P. Werkhoven
- 1 ex. TNO Defensie en Veiligheid, Directie
Directeur Markt, G.D. Klein Baltink
- 1 ex. TNO Defensie en Veiligheid, vestiging Den Haag,
Manager Waarnemingssystemen (operaties), dr. M.W. Leeuw
- 1 ex. TNO Defensie en Veiligheid, vestiging Den Haag,
Manager Beleidsstudies Operationele Analyse &
Informatie Voorziening (operaties), drs. T. de Groot
- 1 ex. TNO Defensie en Veiligheid, vestiging Rijswijk,
Manager Bescherming, Munitie en Wapens (operaties), ir. P.J.M. Elands
- 1 ex. TNO Defensie en Veiligheid, vestiging Soesterberg,
Manager Human Factors (operaties), drs. H.J. Vink
- 1 ex. Maj. M.A. Grisnigt
DS/DOBBP/Operationeel Beleid
- 1 ex. Lkol mr. R.C. Nulkes
DS/IMS/Afd. Navo-EU/Stafofficier Navo,
nucleaire en non-proliferatiezaken
- 1 ex. D.M. van Weel, HDAB
- 1 ex. Drs. E.S.A. Brands, DJZ/IJB
- 1 ex. Lkol A. Solkesz, HDP/DPB
- 1 ex. Maj. R.F.M. Schröder, Las/PBDL/OB
- 1 ex. Maj R. Brunsting Bc, BS/DS/DOBBP/B
- 1 ex. W. Vermeer, DMO/Matlogco
- 1 ex. H.G.B. Reulink, LBBKL/KPU-bedrijf